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Histone H3.3 deposition at E2F-regulated genes is linked to transcription

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The histone variant H3.3 can be incorporated in chromatin independently of DNA synthesis. By imaging using green fluorescent protein-tagged histones, H3.3 deposition has been found to be linked with transcriptional activation. Here, we investigated H3.3 incorporation during G1 progression on cell-cycle-regulated E2F-dependent genes and on some control loci. We transiently transfected resting cells with an expression vector for tagged H3.3 and we analysed its presence by chromatin immunoprecipitation. We found that replication-independent H3.3 deposition occurred on actively transcribed genes, but not on silent loci, thereby confirming its link with transcription. Interestingly, we observed similar levels of H3.3 occupancy on promoters and on the coding regions of the corresponding genes, indicating that H3.3 deposition is not restricted to promoters. Finally, H3.3 occupancy correlated with the presence of transcription-competent RNA polymerase II. Taken together, our results support the hypothesis that H3.3 is incorporated after disruption of nucleosomes mediated by transcription elongation.
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INTRODUCTION

In higher eukaryotes, epigenetic programming has an essential role in development and in the control of cell fate. Epigenetic marks include DNA modifications, the incorporation of specific histone variants and post-translational modifications of nucleosomal histones. Acetylation and phosphorylation are highly dynamic modifications, as they are set up and removed by specific enzymes. In contrast, methylation is more stable, and methylation of K9 of histone H3 has been proposed to stably mark silent heterochromatin (Jenuwein & Allis, 2001). However, there is some clear evidence that histone H3 K9 methylation can be reversed (Nicolas *et al*, 2003; Janicki *et al*, 2004). Although an enzyme able to remove mono- or dimethyl groups from histone H3 K9 has been described recently (Metzger *et al*, 2005), loss of H3 K9 methylation has also been proposed to be mediated by nucleosome exchange (Ahmad & Henikoff, 2002; Janicki *et al*, 2004). Such a replication-independent (RI) nucleosome assembly relies on a specific variant of histone H3, H3.3. H3.3 interacts with a specific deposition machinery (Tagami *et al*, 2004) containing the HIRA protein, which is able to assemble nucleosomes in a DNA synthesis-independent manner (Ray-Gallet *et al*, 2002).

RI deposition of H3.3 occurs during the process of transcriptional activation (Ahmad & Henikoff, 2002; Janicki *et al*, 2004). This has led to the proposal that the presence of histone H3.3 could be an epigenetic imprint of transcriptionally active chromatin (Ahmad & Henikoff, 2002; McKittrick *et al*, 2004). Alternatively, deposition of H3.3 could reflect the *de novo* nucleosome assembly after nucleosome disruption by the progressing RNA polymerase (Ahmad & Henikoff, 2002). Finally, a third hypothesis states that H3.3 deposition could be required to remove pre-existing epigenetic marks (Ahmad & Henikoff, 2002; Janicki *et al*, 2004), thereby correlating with transcriptional competence when these marks are linked to transcriptional silencing.

The E2F-regulated cell-cycle-dependent genes are largely regulated by epigenetic marks. In proliferating cells, classical E2F-responsive genes are activated at the end of G1 phase and the beginning of S phase by the E2F transcription factor. Before the end of G1 or in non-proliferating cells, these genes are repressed by members of the retinoblastoma protein (Rb) family, which is composed of Rb itself, and the related p107 and p130 proteins.

Interestingly, the various transcriptional states of E2F-responsive genes are characterized by specific histone modifications, including acetylation and methylation. Irreversible inactivation of transcription of some E2F-responsive genes is accompanied by epigenetic marks corresponding to heterochromatin, such as the SUV39H1-dependent trimethylation of histone H3 K9 (Narita *et al*, 2003; Ait-Si-Ali *et al*, 2004). In resting cells, some E2F-regulated promoters harbour dimethylated histone H3 K9. During cell-cycle progression, this modification is no longer detectable, concomitant with the appearance of acetylated K9 (Ghosh & Harter, 2003; Nicolas *et al*, 2003).

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To investigate the possible role of histone exchange in this apparent histone demethylation, we analysed the incorporation of the histone H3.3 variant at E2F-regulated genes. By comparing transiently and stably transfected cells, we found that H3.3 is deposited throughout the genes during G1 progression but is specifically maintained on promoters during the cell cycle.

RESULTS AND DISCUSSION

Histone H3 occupancy at the *dhfr* promoter

We showed previously that histone H3 K9 experiences apparent demethylation during G1 progression on the E2F-regulated *dhfr* (dihydrofolate reductase) promoter. This demethylation could be due to nucleosome loss. We thus analysed, by chromatin immunoprecipitation (ChIP), histone H3 occupancy on the *dhfr* promoter during G1 progression (Fig 1B). We observed that histone H3 occupancy was slightly lower on the *dhfr* promoter (DHFR-P) and on the *dhfr* coding region (DHFR-c2) than on heterochromatic sequences (GAPDH-h (glyceraldehyde-3-phosphate dehydrogenase); Ferreira *et al*, 2001) or on an intergenic region in the vicinity of the *dhfr* gene (DHFR-i1), consistent with a decreased histone occupancy due to transcription. However, this very slight decrease cannot account for the apparent histone demethylation on the *dhfr* promoter, which suggests that demethylation cannot be entirely explained by nucleosome loss. This result is consistent with the finding that the *dhfr* promoter is enriched for other histone modifications at the end of G1 (Nicolas *et al*, 2003).

RI histone H3.3 deposition on the DHFR promoter

We next envisioned the possibility that the methylated histone was replaced by unmethylated histone through RI nucleosome assembly. To test this possibility, we intended to detect H3.3 deposition on the *dhfr* promoter during G1 progression. We transiently transfected resting cells with a tagged H3.3 expression vector, to investigate H3.3 deposition independently from S-phase genome-wide deposition (Ahmad & Henikoff, 2002) and from possible maintenance mechanisms. We used an expression vector for H3.3 fused to a double haemagglutinin (HA) tag, the deposition of which can be followed by ChIP (Daury & Trouche, 2003) and which does not lead to H3.3 overexpression (supplementary information 1 online). This tagged H3.3 interacted with HIRA (supplementary information 2 online), indicating that it can be used to monitor RI histone H3.3 deposition.

We transiently transfected the expression vector for the HA-tagged histone in serum-starved NIH3T3 cells (Fig 2A). After 34 h, we added FCS to induce cell-cycle progression. Cells reached S phase by 10 h after serum addition (supplementary information 3 online). The expression of E2F-responsive genes, such as the *dhfr* or *cyclin E* encoding genes, increased by 8 h after serum addition (supplementary information 4 online).

To measure H3.3 deposition before S phase, we carried out ChIP analysis 8 h after serum addition and in the presence of aphidicolin, an inhibitor of DNA polymerase. We measured the amount of the *dhfr* promoter by quantitative PCR (Q-PCR). We detected a significant amount of the *dhfr* promoter in the anti-HA immunoprecipitates compared with the control (Fig 2B). Strikingly, when compared with a DNA sequence derived from the ribosomal phosphoprotein P0 encoding gene (P0), the *dhfr* promoter was specifically enriched in the anti-HA

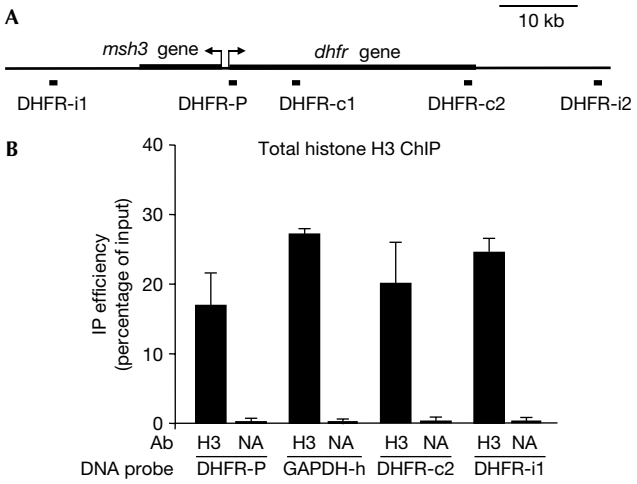

Fig 1 | Histone H3 is not depleted from the dihydrofolate reductase promoter during G1 progression. (**A**) Schematic representation of the mouse *dhfr* (dihydrofolate reductase) locus, with the position of probes detected by quantitative PCR (Q-PCR) in chromatin immunoprecipitation (ChIP) experiments. Note the presence of the *msh3* gene 600 bp upstream of the *dhfr* transcription start site and transcribed in the opposite direction. (**B**) NIH3T3 cells were starved of serum for 48 h and then induced for 8 h with 20% FCS. Cells were then subjected to a ChIP assay using an anti-H3 antibody or no antibody as a control (NA). The amounts of the indicated sequences were measured by Q-PCR. A representative experiment out of three is shown. Ab, antibody.

immunoprecipitates. (Note that the *dhfr* promoter and P0 sequence were analysed in all subsequent experiments as positive and negative controls, respectively.) As a further specificity control, we carried out similar experiments using the H3.1 variant. We found that H3.1 did not undergo significant deposition on the *dhfr* promoter during G1 progression, although it was expressed at levels comparable to H3.3 (Fig 2C) and can be incorporated in chromatin in proliferating cells (Daury & Trouche, 2003). Taken together, these experiments indicate that the *dhfr* promoter can be specifically targeted for RI histone H3.3 deposition during G1 progression, that is, when histone H3 K9 experiences apparent demethylation.

Specificity of H3.3 deposition

We then analysed various DNA regions to investigate the determinants of H3.3 deposition (Fig 2D). First, we did not find any deposition on heterochromatic sequences (GAPDH-h), which thereby provided another negative control. We observed significant H3.3 deposition on two other E2F-regulated promoters (the *cyclin E* (cycE-P) and *cdc6* (cdc6-P) promoters), and on the β*actin* promoter (βactin-P), which is an immediate-early gene not regulated by E2F, indicating that the targeted deposition of histone H3.3 is not specific for the *dhfr* promoter or for E2F-responsive promoters.

We also studied H3.3 incorporation along the *dhfr* gene. We found that two probes in the transcribed regions of *dhfr* (DHFR-c1 and DHFR-c2) were almost as enriched as the *dhfr* promoter in H3.3 immunoprecipitates, whereas two probes outside the transcribed region (DHFR-i1 and DHFR-i2) were enriched only to background levels. This result indicates that H3.3 deposition

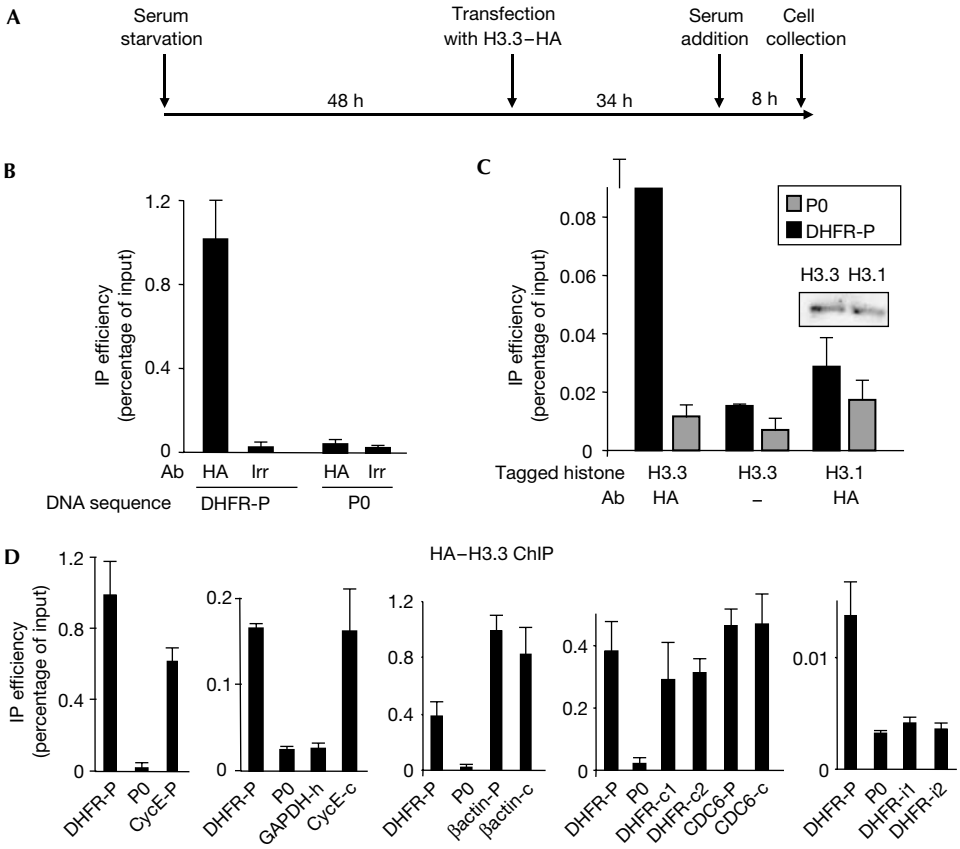

Fig 2 | Study of histone H3.3 deposition during G1 progression. (**A**) Description of the experimental procedure. NIH3T3 cells (400,000 cells/10 cm dish, six dishes per immunoprecipitation) were starved in 0.5% FCS for 48 h and then transiently transfected with 30 μg of the histone H3.3 expression vector per dish. At 34 h after transfection, cells were induced using 20% FCS and collected for chromatin immunoprecipitation (ChIP) 8 h later. (**B**) NIH3T3 cells, treated as described in (**A**), were subjected to a ChIP assay using either the anti-haemagglutinin antibody (HA) or the anti-Flag antibody (Irr) as a control. The amounts of the *dhfr* (dihydrofolate reductase) promoter (DHFR-P), and P0 sequences were measured by quantitative PCR (Q-PCR). A representative experiment out of ten is shown. Ab, antibody. (**C**) Same as (**B**), except that NIH3T3 cells were transfected with either the H3.3–HA or the H3.1–HA expression vector. A representative experiment out of two is shown. (**D**) Same as (**B**), except that the amounts of the indicated sequences were measured by Q-PCR. A representative experiment out of three is shown.

was observed to a similar extent throughout the *dhfr* transcribed region. Moreover, in similar experiments, we also observed H3.3 deposition on probes derived from the coding regions of the *cyclin E* (cycE-c), the *cdc 6* (cdc6-c) and the β*actin* (βactin-c) genes and far away from the promoters, which suggested that in these cases also deposition of H3.3 occurred on the whole transcribed region.

Histone H3.3 occupancy in stably expressing cells

Our results are in contrast to the recent finding that H3.3 specifically marks promoters (Chow *et al*, 2005). Chow *et al* used stably expressing cells in which histone H3.3 occupancy reflects both its deposition and its maintenance during the cell cycle. We raised a pool of NIH3T3 cells stably expressing H3.3–HA, and analysed the presence of H3.3 by ChIP during G1 progression (Fig 3). We found that H3.3 was present in all the sequences we tested, including intergenic and heterochromatic sequences (P0, DHFR-i1 (Fig 3) and heterochromatic GAPDH (data not shown)), which are not targeted for H3.3 deposition during G1 progression (compare with Fig 2). This result probably reflects the genome-wide histone H3.3 incorporation in S phase, as already observed by Ahmad & Henikoff (2002). Moreover, above this background incorporation, the *dhfr* and β*actin* promoters were slightly more enriched than their corresponding transcribed regions (compare DHFR-P with DHFR-c1, and βactin-P with βactin-c), confirming that histone H3.3 occupancy is higher at some promoters, as already observed by Chow *et al* (2005). ChIP analysis using quiescent cells gave similar results (supplementary information 5 online), indicating that transcription-linked deposition during G1 progression mainly resulted in the exchange of previously bound H3.3 by newly synthesized H3.3. Taken together, our data indicate that histone H3.3 deposition occurred in G1 throughout the transcribed regions (Fig 2) and that H3.3 is specifically enriched on the *dhfr* and β*actin* promoters when expressed throughout the cell cycle (Fig 3).

Deposition of histone H3.3 correlates with transcription

It has been proposed that progression of the RNA polymerase II (RNAP) could lead to nucleosome disruption and the subsequent

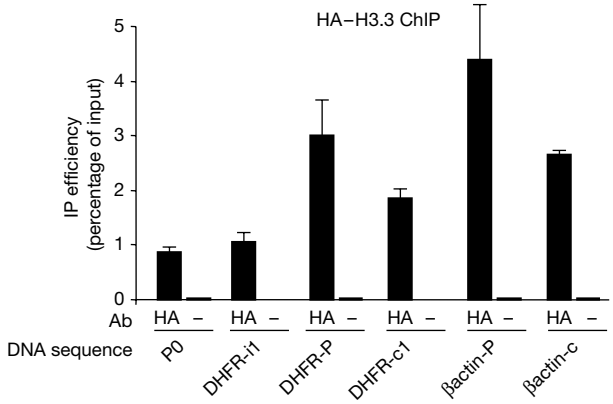

Fig 3 | Analysis of histone H3.3 presence in stably expressing cells. NIH3T3 cells stably expressing tagged H3.3 were starved of serum for 48 h and then re-induced for 8 h with 20% FCS and subjected to a chromatin immunoprecipitation (ChIP) assay using the anti-haemagglutinin antibody (HA) or no antibody as a control. The amounts of the indicated sequences were measured by quantitative PCR. A representative experiment out of three is shown. Ab, antibody.

deposition of H3.3-containing nucleosomes (Ahmad & Henikoff, 2002). Our results on H3.3 deposition (Fig 2) are entirely consistent with such a hypothesis. To test this possibility, we investigated whether H3.3 deposition correlates with the presence of transcribing RNAP. Unfortunately, we could not get a significant ChIP signal with the antibody that recognized RNAP phosphorylated on S2, which is the elongating RNAP (data not shown). We thus carried out ChIP analysis with anti-RNAP antibody (Fig 4A) and anti-phospho-S5 RNAP antibody (S5P-RNAP; Fig 4B). This latter antibody recognizes RNAP after transcription initiation.

We observed that both antibodies immunoprecipitated a higher amount of E2F-responsive promoters than of the corresponding coding sequences (compare left and right panels), probably reflecting the longer time spent by RNAP and its S5P-RNAP on the promoter. The βactin promoter and coding region were enriched at equivalent levels in both RNAP and S5P-RNAP. A comparison of RNAP presence in the coding regions of transcribed genes, which reflects transcription (Sandoval *et al*, 2004), indicates that the β*actin* gene is probably two times more transcribed than the three E2F-regulated promoters. Finally, as expected, probes derived from intergenic sequences (DHFR-i1 and DHFR-i2) or from the heterochromatic pseudogenes (GAPDH-h) were only marginally enriched in RNAP immunoprecipitates, as was the P0 sequence (indicating that although this gene is a housekeeping gene, it is transcribed at relatively low levels). Strikingly, we observed the existence of a correlation between transcription and H3.3 deposition. Indeed, all sequences with background levels of RNAP were not enriched in H3.3 immunoprecipitates (DHFR-i1, DHFR-i2, GAPDH-h and P0), whereas all sequences derived from actively transcribed genes were significantly enriched (DHFR-P, DHFR-c1, DHFR-c2, CDC6-P, CDC6-c, Cyc E-c, βactin-P and βactin-c; compare Figs 4 and 2D). Moreover, among these sequences, the most transcribed gene (β*actin*) experienced the highest amount of H3.3 incorporation.

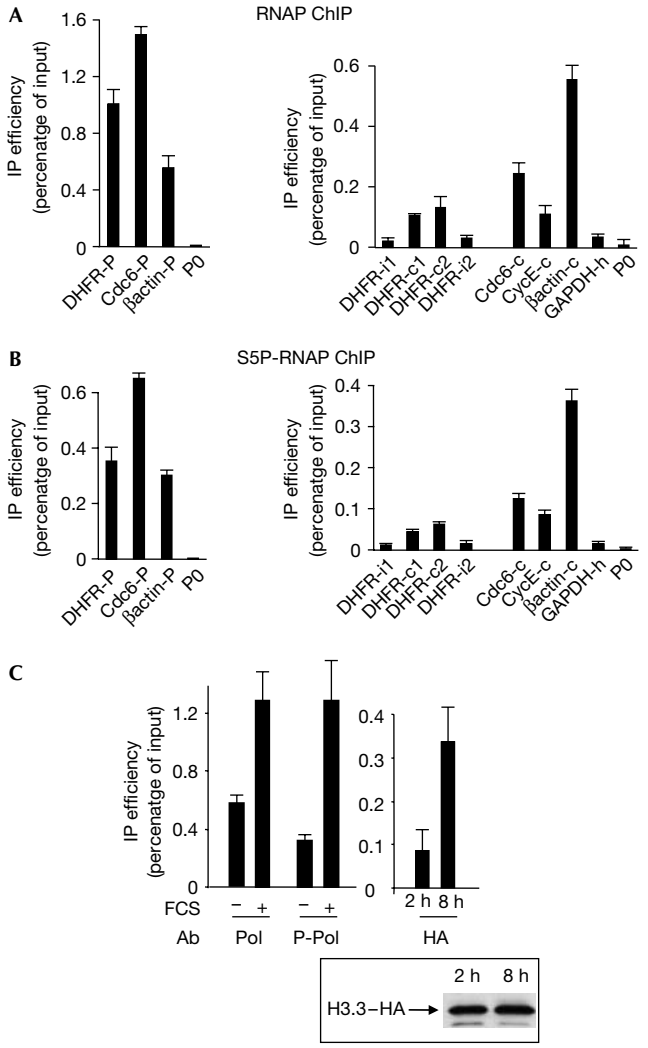

Fig 4 | Deposition of H3.3 correlates with transcription. NIH3T3 cells were starved of serum for 48 h and then re-induced for 8 h with 20% FCS and subjected to a chromatin immunoprecipitation (ChIP) assay using an anti-pol II antibody (**A**) or an anti-S5-phosphorylated pol II antibody (**B**). The amounts of the indicated sequences were measured by quantitative PCR (Q-PCR). A representative experiment out of three is shown. (**C**) Left panel: NIH3T3 cells were starved of serum for 48 h and then re-induced or not, as indicated, with 20% FCS for 8 h. Right panel: NIH3T3 cells were transfected with the H3.3–haemagglutinin (HA) expression vector and re-induced for 2 or 8 h with 20% FCS, as indicated. Cells were then subjected to a ChIP assay using an anti-RNA polymerase II antibody (RNAP), anti-S5 phosphorylated RNAP antibody (S5P-RNAP; left panel) or an anti-HA antibody (right panel). The amount of *dhfr* (dihydrofolate reductase) promoter was measured by Q-PCR. A representative experiment out of three is shown. Also shown is a western blot monitoring H3.3–HA expression. Note that in the control we induced cells with serum for 2 h because exogenous histone expression is very low in uninduced cells (data not shown). Ab, antibody.

Finally, if deposition of H3.3 is linked to transcription, then it should increase when transcription increases. We thus compared H3.3 incorporation on the *dhfr* promoter before and after the process of transcriptional activation. We found that 8 h after serum stimulation, both RNAP presence and H3.3 incorporation had increased on the *dhfr* promoter (Fig 4C). Thus, taken together, these results indicate that deposition of H3.3 is tightly linked to transcription.

What could be the link between H3.3 deposition and transcription? H3.3 deposition could occur on actively transcribed genes to replace nucleosomes disrupted by RNAP progression (Ahmad & Henikoff, 2002). Our results are entirely consistent with such a mechanism, because we observed deposition in the transcribed regions of various genes and this deposition largely correlated with transcription levels. Similar results were recently described in Drosophilae (Schwartz & Ahmad, 2005). Interestingly, our data indicate that, whereas H3.3 deposition is similar on promoters and on the corresponding transcribed regions during G1 progression, at least two promoters (*dhfr* and β*actin*) are slightly but significantly enriched in H3.3 immunoprecipitates when H3.3 is expressed throughout the cell cycle (Fig 3). A likely possibility is that this enrichment is due to abortive rounds of transcription initiation that would occur on these promoters at specific cell-cycle stages. If this hypothesis is correct, then the higher H3.3 presence at these promoters could also merely be a consequence of transcription. Alternatively, H3.3 occupancy could be actively maintained on these promoters during the cell cycle by specific mechanisms, the identity of which remains elusive. Because of the potential role of H3.3 in transcriptional regulation, it will undoubtedly be important to confirm these results for other promoters and to investigate whether H3.3 presence is actively maintained on promoters during the cell cycle.

Although we cannot rule out so far the possibility that H3.3 deposition is only a consequence of the process of transcription, our results raise the question of its role in transcriptional activation. H3.3 deposition can be important to remove histone K9 methylation, a negative mark for transcription. Indeed, the *dhfr* promoter experiences histone H3 K9 demethylation, H3.3 deposition and histone H3 K9 acetylation during G1 progression (Nicolas *et al*, 2003). As H3.3 is specifically enriched on some promoters, another possibility, although not exclusive, is that its presence functions as an epigenetic imprint specifying transcription-competent chromatin. Whether H3.3-containing nucleosomes have some unique properties deserves to be investigated.

METHODS

Cell culture and transfections. NIH3T3 cells were grown in DMEM supplemented with antibiotics, 1% sodium pyruvate and 10% FCS (all from Invitrogen, Carlsbad, CA, USA). Cells were transfected using the Transfast reagent (Promega, Madison, WI, USA) according to the manufacturer's instructions. For synchronization, cells were starved for 48 h in DMEM supplemented with 0.5% FCS and re-induced by the addition of 20% FCS. pBos H3.3–HA, pBos H3.1–HA and empty vector have been described previously (Daury & Trouche, 2003). For establishment of a pool of cells stably expressing H3.3, NIH3T3 cells were transfected by calcium phosphate co-precipitation with the pBos H3.3–HA expression vector and pcDNA3.1 and selected using G418 (300 μg/ml). Cells were then tested by western blot for the expression of the tagged histone.

Chromatin immunoprecipitation. ChIP analysis was carried out essentially as described (Ferreira *et al*, 2001). Briefly, cells were treated with formaldehyde for crosslinking and cell extracts were sonicated to obtain DNA fragments of 1 and 2 kb. Immunoprecipitation was carried out with 10 μg of anti-HA 16B12 antibody (Covance Research Products, Cumberland, VA, USA) or anti-Flag M2 antibody (Sigma, St Louis, MO, USA) as a control antibody, anti-RNAP antibody (CTD4H8; Upstate Biotechnologies, Charlottesville, VA, USA), anti-phospho-S5 RNAP antibody (H14; Covance), anti-histone H3 (gift from Dr S. Müller) or without antibody. After crosslink reversion, the immunoprecipitated DNA and input DNA were purified on a GFX™ column (GE Healthcare Biosciences, Buckinghamshire, UK) and quantified by real-time PCR (Q-PCR).

Quantitative PCR. Real-time PCR was carried out using Sybr green I (Sigma) and Platinium Quantitative PCR Supermix-UDG (Invitrogen; except for the cyclin E promoter, which was amplified using the quantitect PCR kit (Qiagen, Hilden, Germany)) on an ICyclerQ™ (Bio-Rad, Hercules, CA, USA) real-time PCR device. Each experiment included a standard curve. PCR conditions and oligonucleotide sequences and the position of oligonucleotides relative to the transcription start site are described in supplementary information 6 online. Samples were analysed in triplicate, and the mean and standard deviation were calculated.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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